Melanocortin-1 Receptor Signaling Markedly Induces the Expression of the NR4A Nuclear Receptor Subgroup in Melanocytic Cells*

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The melanocortin-1 receptor (MC1R) is a G-protein-coupled receptor expressed primarily in melanocytes and is known to play a pivotal role in the regulation of pigmentation in mammals. In humans MC1R has been found to be highly polymorphic with several functional variants associated with the phenotype of red hair color and fair skin, cutaneous UV sensitivity, and increased risk of developing melanoma and non-melanoma skin cancer. Recent evidence suggests that MC1R plays a photo-protective role in melanocytes in response to UV irradiation. Relatively few genetic targets of MC1R signaling have been identified independent of the pigmentation pathway. Here we show that MC1R signaling in B16 mouse melanoma cells and primary human melanocytes rapidly, and transiently, induces the transcription of the NR4A subfamily of orphan nuclear receptors. Furthermore, primary human melanocytes harboring homozygous RHC variant MC1R alleles exhibited an impaired induction of NR4A genes in response to the potent MC1R agonist (Nle4,D-Phe7)-α-melanocyte-stimulating hormone. Using small interference RNA-mediated attenuation of NR4A1 and NR4A2 expression in melanocytes, the ability to remove cyclobutane pyrimidine dimers following UV irradiation appeared to be impaired in the context of MC1R signaling. These data identify the NR4A receptor family as potential mediators of an MC1R-coordinated DNA damage response to UV exposure in melanocytic cells.

It has long been recognized that individuals with fair skin and poor tanning ability have an increased incidence of melanoma and non-melanoma skin cancer (1–3). Cutaneous pigmentation in humans is the result of melanin synthesis by epidermal melanocytes within specialized organelles termed melanosomes, which are transferred to surrounding keratinocytes (4).

Human pigmentation is a polygenic trait with >60 genetic loci identified so far (5, 6). Efforts over the last decade to understand the genetic basis of human pigmentary traits have revealed the melanocortin-1 receptor (MC1R)[5] as a key determinant of the wide phenotypic diversity in mammals (7–9). MC1R is a seven-transmembrane G-protein-coupled receptor that has been found to be highly polymorphic in Caucasian individuals, with a number of functional variants strongly associating with a phenotype of red hair, fair skin, and poor tanning ability often referred to as the Red Hair Color (RHC) phenotype (7). MC1R preferentially binds the pro-opiomelanocortin-derived peptides α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone, which leads to an elevation in intracellular cAMP levels (10). RHC variant MC1Rs have been demonstrated to elicit weak cAMP responses as a result of reduced receptor coupling (2, 11–13) or abnormal receptor localization (2, 12–15). Molecular analysis has revealed that MC1R signaling modulates the expression and function of the microphthalmia-associated transcription factor, a key regulator of pigmentation genes, suggesting that this pathway underlies MC1R coordination of melanogenesis (16). Accumulating evidence suggests the photo-protective effect of MC1R signaling goes beyond quantitative and qualitative changes in melanin synthesis and extends to protection against UV-induced genomic damage via the induction of nucleotide excision repair mechanisms (17–19). Although variant MC1R is anticipated to have a significant effect on melanocyte gene expression, few downstream transcriptional targets of MC1R signaling have been identified to date (20–22) and the differential transcriptional response between wild-type and RHC signaling remains poorly understood.

The NR4A genes are a subfamily of nuclear receptors that includes NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (NOR-1) in mammals. The nuclear receptor superfamily of transcriptional regulators includes the classical steroid receptors, the thyroid and retinoid receptors, and a large subgroup of receptors referred to as "orphan receptors" as no natural or synthetic hormone/ligands have been identified to date (23, 24).

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[5] The abbreviations used are: MC1R, melanocortin-1 receptor; α-MSH, α-melanocyte-stimulating hormone; NDP-MSH, (Nle4,D-Phe7)-α-MSH; NR, nuclear receptor; q-RT-PCR, quantitative real-time PCR; RHC, red hair color; UVB, ultraviolet-B radiation; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; siRNA, small interference RNA; EMSA, electrophoretic mobility shift assay; WT, wild type; CPD, cyclobutane pyrimidine.

The NR4A subfamily belongs to the orphan receptor subgroup, and crystal structure analysis suggests these receptors may be true orphan receptors that are unable to accommodate molecules within their ligand binding domain in the same manner as traditional NR ligand binding domains (25, 26). The NR4A subfamily was first identified as early response genes for growth factors (27, 28). All three NR4A receptors bind to DNA consensus elements termed NBREs as monomers or to a palindromic NurRE sequence as homo- or heterodimers (29).

NR4A genes have been implicated in a wide range of biological processes including proliferation, differentiation, apoptosis, and tissue remodeling and in pathologies such as neurological disorders including Parkinson disease, schizophrenia and manic depression, inflammatory and cardiovascular disease, and cancer (25, 30). Expression and function of the NR4A family of nuclear receptors are influenced by a wide range of signaling pathways that utilize a number of signaling cascades to generate a functional output, including cAMP, MEK/extracellular signal-regulated kinase (ERK), p38/MAPK, protein kinase C, and Ca^{2+} (25, 31–33). Recent reports demonstrate the rapid induction of NR4A genes in macrophage cells in response to inflammatory stimuli (34) and in hepatic and skeletal muscle tissues where they coordinate pathways involved in glucose and lipid homeostasis (31–33). Interestingly, reports demonstrating the activation of NR4A proteins by the purine anti-metabolite 6-mercaptopurine and related compounds suggest that this gene family may be involved in the genotoxic stress response (35, 36).

The induced expression of NR4A1 has been reported previously in melanoma cells in response to interleukin-1β signaling (37) and has recently been linked to melanoma apoptotic sensitivity (38). The expression and function of the NR4A2 and NR4A3 genes in melanocytic cells have not been reported to date. Here we report that all three NR4A receptors are induced and function immediately downstream of MC1R signaling in melanocytic cells. Furthermore, we demonstrate the impaired induction of the NR4A gene family in primary human melanocytes of MC1R variant genotype. This observation reveals the NR4A gene family as one of the immediate transcriptional targets of MC1R signaling in melanocytic cells. Moreover, the functional role of this nuclear receptor subfamily in melanocyte gene expression remains to be explored.

MATERIALS AND METHODS

Cell Culture, UV Irradiation, and Transfection Analysis—B16 melanoma cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and penicillin (20 units/ml)/streptomycin (20 g/ml). Transfections using the pGL2B-NurRE luciferase reporter (39) were performed in 24-well tissue culture dishes with 0.8 mg of DNA/well using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested and analyzed as described previously (31, 40).

Primary human melanocytes (QF278, 840, 731, 863, 955, and 1093) were cultured using established protocols (41). Primary human melanocytes derived from differentiated melanoblasts (QF1170 and 1160) were cultured and differentiated as described previously (42). Differentiating melanocytes were grown on coverslips for 3 days prior to UV irradiation experiments, and cells were transfected with siRNA reagents 24 h prior to UV irradiation. siRNA reagents were transfected into cells using Lipofectamine 2000 according to the manufacturer's recommendation to a final concentration of 50 nM. siRNA reagents were: negative control (Ambion), NR4A1 (1:1 pooled ID41824 and ID41752; Ambion), and NR4A2 (Smartpool; Dharmacon). Following a 4-h preincubation in medium supplemented with either vehicle or NDP-MSH (10 nM), cells were irradiated with 25 mJ cm^{-2} UVB in phosphate-buffered saline as described previously (41). Following irradiation, cells were maintained in vehicle or NDP-MSH-containing medium for a further 24 h, at which point cells were fixed in 4% paraformaldehyde and analyzed by immunofluorescence.

RNA Analysis and Real-time PCR—Total RNA was extracted from melanoma cell lines using TRI Reagent (Sigma-Aldrich) followed by DNase treatment and further purification using the RNeasy RNA extraction kit (Qiagen) described previously (31, 40). Complementary DNA was synthesized from 1 μg of total RNA using a random hexamer and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Target cDNA levels were analyzed by q-RT-PCR using SYBR Green or TaqMan reagent (ABI) as described previously (31, 40). Primer sequences are available by request.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared using B16 cells as described previously (43). EMSA was performed using radiolabeled annealed oligonucleotides containing the NBRE consensus element (44). Oligonucleotide sequences were 5'-gggatcctcgtgcgaaaaggtcaagcg-cta-3' (upper strand), 5'-ggttcctggcatttgaggtaaatatcacta-3' (lower strand).

UV Irradiation of Cultured Cells and Immunofluorescence—Primary human melanoblasts were cultured on coverslips and differentiated into melanocytes and manipulated as described above. Immunofluorescence was performed on paraformaldehyde-fixed cells to determine nuclear CDP content using an anti-cyclobutane pyrimidine dimer antibody (TDM-2; MBL International Corp.) according to the manufacturer's recommended protocol. For quantification six to eight representative pictures per coverslip were taken and analyzed in Adobe Photoshop for fluorescence intensity (grey scale). Images were obtained on an Olympus BX-51 epifluorescence microscope with a DP-70 camera.

Statistical Analysis—Statistical analysis was performed on a minimum of three independent experiments using a ratio *t* test or one-way analysis of variance followed by a Tukey's post-test using GraphPad-PRISM software.

RESULTS

Induction of NR4A Gene Expression by MC1R Signaling in B16 Melanoma—We first analyzed the NR4A class of nuclear receptors as potential target genes of MC1R signaling using B16 mouse melanoma cells. B16 cells were chosen as they express wild-type MC1R with an intact downstream signaling pathway (45, 46). As NR4A genes are known to be serum-responsive (25, 26), B16 cells were preincubated for 2 h in serum-free medium, at which point cells were treated with vehicle or 10 nM NDP-MSH and RNA harvested at the indicated time points over 36 h.
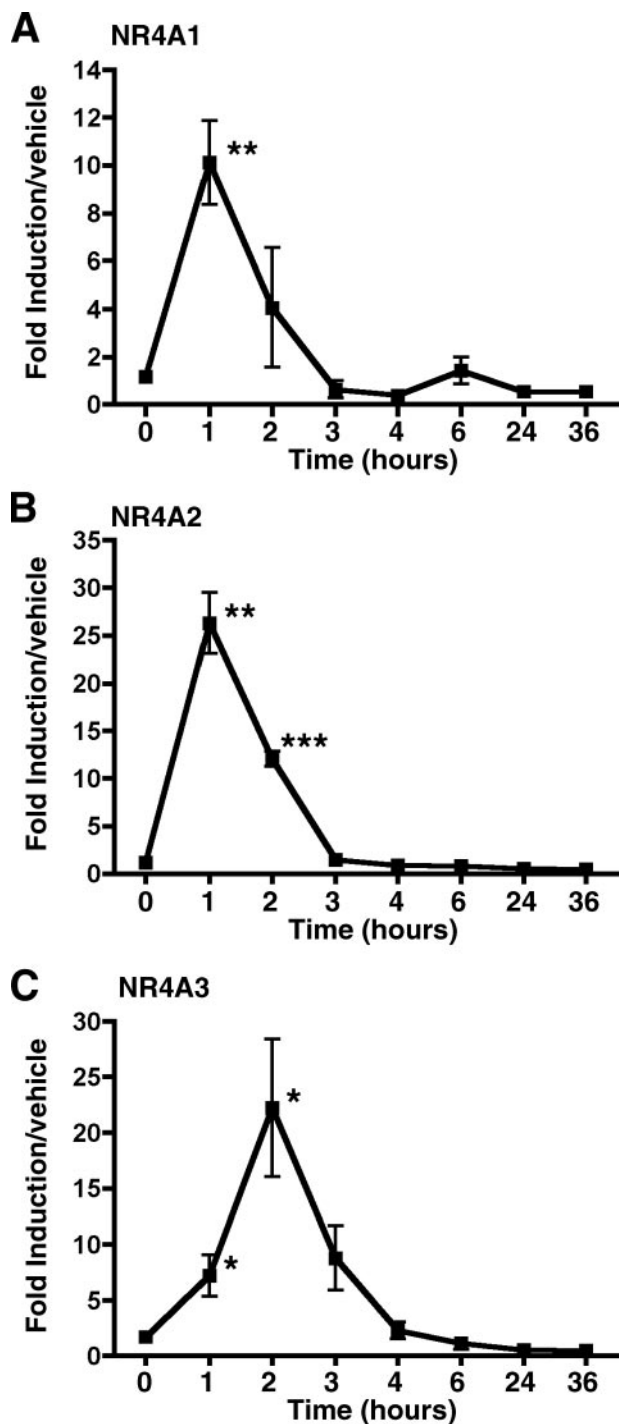
FIGURE 1. **NR4A genes are induced by MC1R signaling in B16 melanoma cells.** *A–C*, B16 mouse melanoma cells were preincubated for 2 h in serum-free medium, at which point cells were treated with vehicle or 10 nM NDP-MSH and RNA harvested as indicated over a 36-h time course. NR4A gene expression analysis was performed by q-RT-PCR for expression of NR4A1 (*A*), NR4A2 (*B*), or NR4A3 (*C*). NDP-MSH-induced q-RT-PCR results were normalized against the glyceraldehyde-3-phosphate dehydrogenase control gene and converted to -fold induction relative to vehicle treatment for each time point. Data represent the mean ± S.E. of three independent experiments. Statistical analysis was performed using analysis of variance with a Tukey's post-test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

FIGURE 2. **Increased NR4A activity by MC1R signaling in B16 melanoma cells.** *A*, B16 cells were transfected with the pGL2B-NurRE luciferase reporter construct for 22 h, at which point the cells were supplemented with medium containing vehicle or NDP-MSH (10 nM) following a 2-h preincubation in serum-free medium. Cells were harvested and analyzed for luciferase expression at 2, 4, 6, and 24 h post ligand administration. NDP-MSH-induced luciferase activity was normalized to vehicle-treated cells for given time points. Data represent the mean ± S.E. of three independent experiments. *B*, EMSA analysis was used to detect NR4A DNA binding activities in nuclear extracts obtained from B16 cells treated with vehicle (*V*), NDP-MSH (*M*), or forskolin (*F*) for 8 h; *FP*, free probe. A representative experiment from three independent experiments is shown.

Time point RNA samples were analyzed by real-time PCR for expression of each NR4A family member. This analysis revealed that the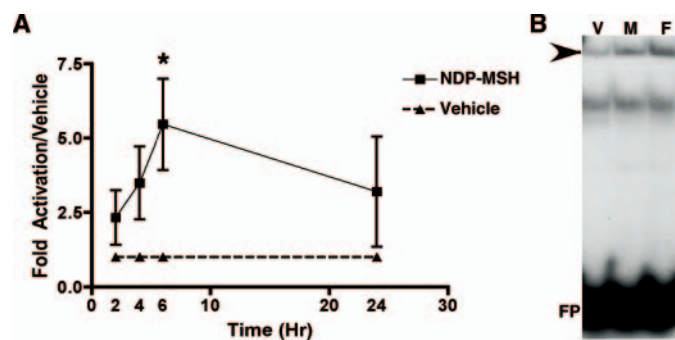 mRNAs encoding the NR4A1–3 genes were rapidly and transiently induced following NDP-MSH treatment in B16 melanoma cells (Fig. 1). More specifically, NR4A1 and NR4A2 showed maximal induction at 1 h, while maximal induction of NR4A3 occurred at 2 h post NDP-MSH treatment.

Analysis of NR4A function in B16 cells in response to MC1R signaling was performed using a transiently transfected NR4A reporter construct containing the NurRE consensus element upstream of the luciferase gene (39). Increased luciferase activity was detected in B16 cells treated with NDP-MSH compared with control cells, with maximal activity detected after 6 h in experiments performed here (Fig. 2*A*). Furthermore, luciferase activity was returning to basal levels 24 h post NDP-MSH stimulation, suggesting the NR4A proteins are functioning within the first 24 h after MC1R activation.

Direct detection of NR4A DNA binding activity in nuclear extracts generated from B16 cells 8 h after vehicle, NDP-MSH, or forskolin treatment was performed by EMSA. Comparison of DNA-protein complexes bound to an NBRE consensus element (44) in these studies demonstrated an increased binding activity in both NDP-MSH- and forskolin-treated cells when compared with the vehicle control (Fig. 2*B*). These data confirm increased NR4A mRNA and protein expression and functional activity in B16 cells occurs immediately downstream of MC1R signaling.

Cross-talk between the NR4A Nuclear Receptor and MC1R Signaling Cascades in B16 Melanoma Cells—Although MC1R signaling functions primarily via the stimulation of adenylate cyclase resulting in the production of cAMP, emerging evidence suggests that a number of other signaling cascades are also activated by MC1R in melanocytic cells (10, 41, 47, 48). Moreover, the NR4A subgroup has been shown to be targets of cAMP signaling in a number of cell types (25, 26). To identify the signaling cascades involved in MC1R-mediated induction of NR4A genes, we stimulated B16 melanoma cells with vehicle or NDP-MSH in the presence of a number of specific signaling protein inhibitors, including H89 (protein kinase A), GF10933X (protein kinase C), SB203580 (p38/MAPK), and
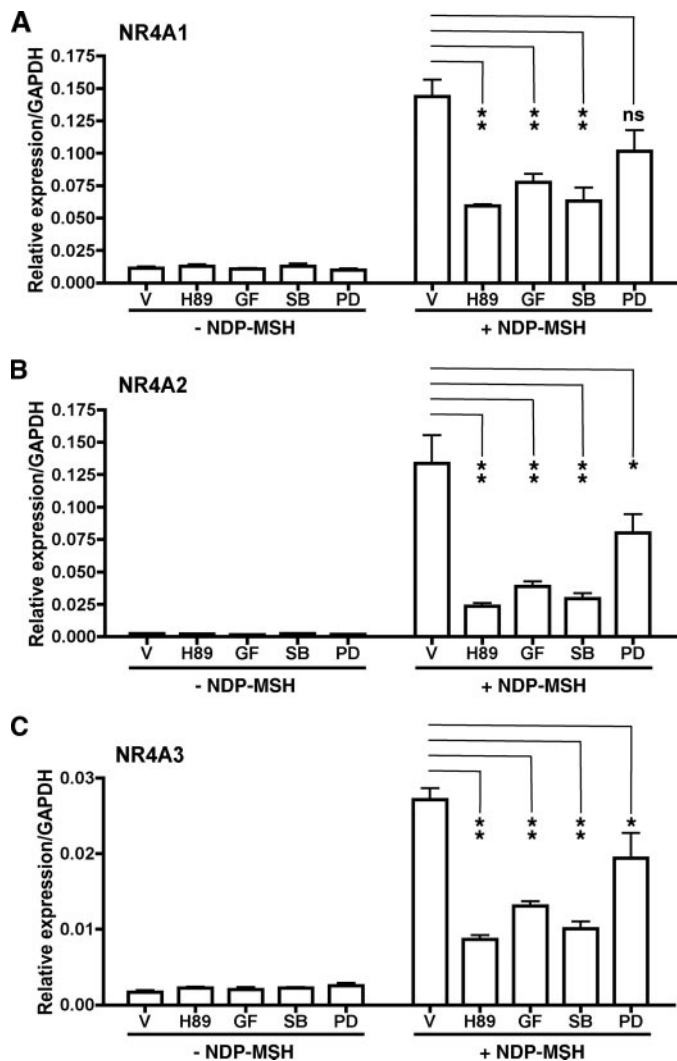
FIGURE 3. **MC1R signaling induces NR4A gene expression by multiple signaling cascades.** *A–C*, mouse B16 cells were treated with 10 nM NDP-MSH in the presence of DMSO (*V*, vehicle control), H89 (10 μM), GF10933X (*GF*, 10 μM), SB203580 (*SB*, 20 μM), or PD98059 (*PD*, 20 μM) for 1 h following a 2-h preincubation in serum-free medium. Total cellular RNA was isolated and analyzed by q-RT-PCR for NR4A1 (*A*), NR4A2 (*B*), and NR4A3 (*C*) expression. Expression was determined relative to glyceraldehyde-3-phosphate dehydrogenase and represents the mean ± S.E. of three independent experiments. Statistical analysis was performed using analysis of variance with a Tukey's post-test (**, $p < 0.01$; *, $p < 0.05$). *ns*, not significant.

PD98059 (MEK1/2). As shown in Fig. 3, NDP-MSH-mediated induction of all three NR4A genes was observed in the presence of the vehicle control (DMSO) while attenuation of this response was observed to varying degrees with the inhibitor compounds, suggesting multiple signaling cascades are activated by MC1R (Fig. 3).

Effect of RHC Variants on MC1R-mediated Induction of NR4A Genes—Given the importance of the MC1R signaling pathway in human pigmentation and UV protection, we determined whether the NR4A genes are similarly induced by NDP-MSH in human melanocytes. To this end, primary human melanocytes of defined MC1R genotypes were treated with NDP-MSH or vehicle and analyzed for NR4A induction at 1- and 3-h time points following administration. Previous reports have demonstrated the ability of wild-type consensus MC1R

alleles to stimulate the cAMP pathway in response to NDP-MSH, an effect that is significantly attenuated in the context of homozygous RHC variant alleles (9, 13, 49). Three melanocyte strains homozygous for the wild-type MC1R exhibited a marked induction of the NR4A2 gene in response to NDP-MSH above basal levels (Fig. 4*B*). By contrast, the levels of NR4A2 expression in homozygous RHC variant MC1R strains were significantly impaired (Fig. 4*B*). A comparison of pooled expression data in these melanocyte strains revealed the differential induction of NR4A2 expression between the wild-type- and RHC-expressing melanocytes in response to NDP-MSH was statistically significant (Fig. 4*E*). Induction of the NR4A1 and NR4A3 subtypes in response to NDP-MSH stimulation was also apparent in wild-type MC1R-expressing melanocytes, whereas the levels of induced NR4A1 or NR4A3 expression were lower in the context of RHC variant MC1R (Fig. 4, *A* and *C*). Comparison between pooled WT and RHC expression data for the NR4A1 and NR4A3 genes revealed a similar trend in which induction of these family members was reduced in RHC variant melanocytes relative to the induction levels observed in the context of WT MC1R (Fig. 4, *D* and *F*). In all melanocyte strains used in this study NR4A gene expression returned to basal levels 3 h post NDP-MSH stimulation (data not shown).

NR4A Role in MC1R-mediated DNA Repair in Melanocytes—Recent evidence has shown that MC1R function protects against DNA damage independently of pigmentation phenotype. Given the immediacy of NR4A induction by MC1R signaling, we decided to investigate the effect of attenuated NR4A1/2 expression on the photo-protective activity of MC1R. Cyclobutane pyrimidine (CPD) dimers are a hallmark DNA lesion induced by ultraviolet radiation. Previous investigations have revealed that MC1R signaling in melanocytes facilitates the clearance of UVB-induced cyclobutane pyrimidine dimers, pointing to an important role for MC1R in protection against UV-induced mutations that may lead to malignancy (17–19). Accordingly, we chose to examine the effect of loss of NR4A gene expression on the ability of MC1R signaling to promote repair of CPD lesions. First we demonstrated that NR4A1/2 expression induced by MC1R signaling could be attenuated in primary human melanocytes using NR4A1/NR4A2-specific siRNAs. Although transfection with a negative siRNA did not prevent MC1R-mediated induction of NR4A1 and NR4A2 mRNA, the specific siRNAs effectively attenuated induction of these genes as assessed by real-time PCR (Fig. 5, *A* and *B*). Dual NR4A1 and NR4A2 knock down was employed in these studies to avoid potential redundancy between these closely related receptors. The ability of melanocytes treated in this manner to remove UVB-induced CPDs in the presence or absence of NDP-MSH was subsequently investigated.

Primary human melanocytes were transfected with either negative or pooled NR4A1/2 siRNAs 24 h prior to UVB irradiation. Following a 4-h preincubation with medium containing either vehicle or NDP-MSH, the melanocytes were irradiated with 25 mJ cm^{-2}, after which they were maintained in vehicle- or NDP-MSH-containing medium for a further 24 h. Visualization of nuclear CPD content in these experiments by immunofluorescence revealed that NDP-MSH-treated cells exhibited less fluorescence than vehicle-treated cells in the presence of a

A NR4A1

B NR4A2
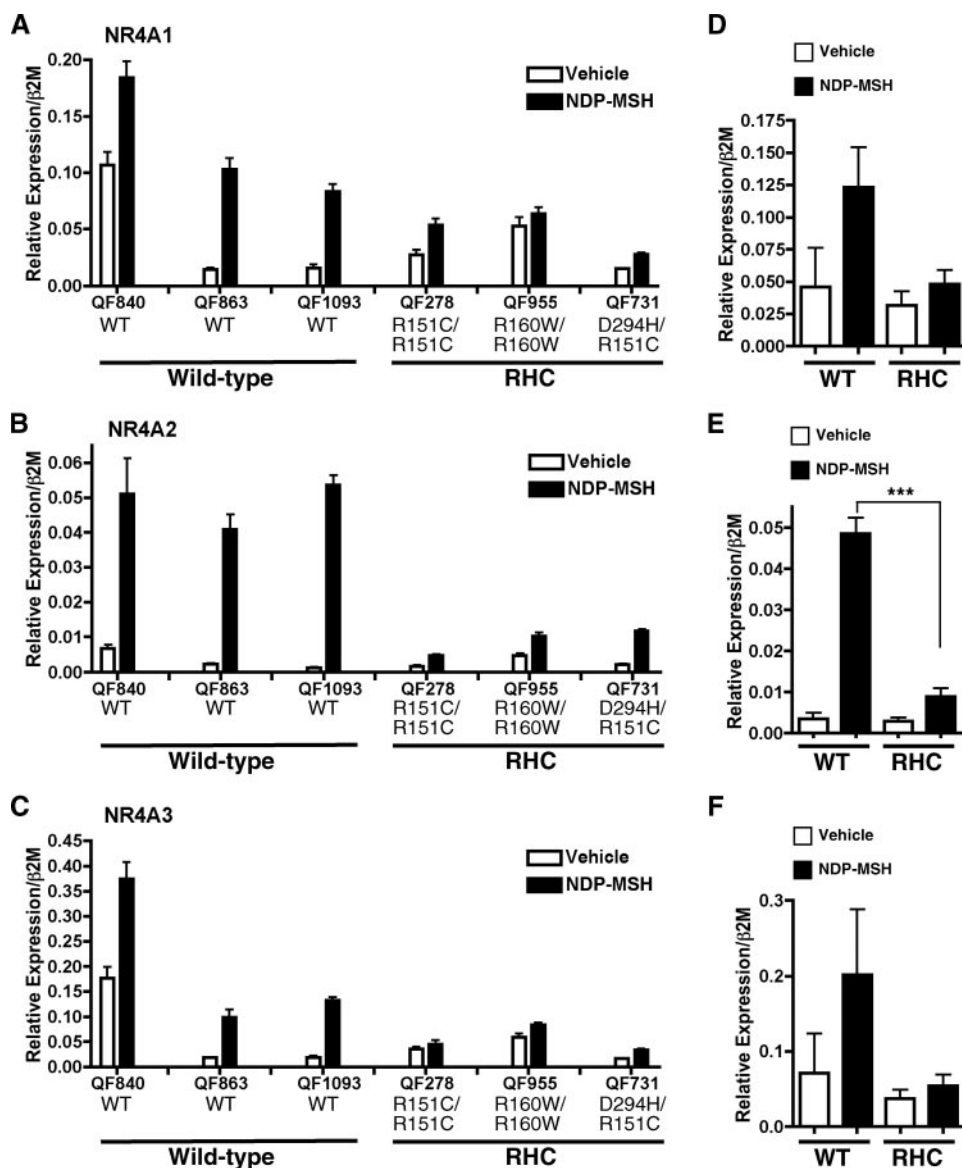
C NR4A3

D

E

F

FIGURE 4. **Human RHC variant MC1R alleles exhibit impaired induction of NR4A expression.** *A–C*, primary human melanocytes were cultured and administered vehicle or NDP-MSH (10 nM) for 1 h. NR4A expression was analyzed by q-RT-PCR (*A–C*). Data shown are mean ± S.D. from a single experiment performed for each cell strain. *D–F*, expression data from *A–C* were pooled to compare NR4A expression levels between WT and RHC variant melanocytes for NR4A1 (*D*), NR4A2 (*E*), and NR4A3 (*F*). Expression levels are shown for three independent WT strains (*QF840*, *QF863*, and *QF1093*) and three independent RHC variant strains (*QF278* (R151C homozygous), *QF955* (R160W homozygous), and *QF731* (R151C/D294H compound heterozygous)). Statistical analysis was performed using analysis of variance with a Tukey's post test (***, $p < 0.001$).

which NR4A1/2 had been knocked down. Moreover, there was as significant difference in the relative CPD content between negative siRNA- and NR4A1/2 siRNA-treated melanocytes in the presence of NDP-MSH. These data confirm the ability of MC1R signaling to mediate the repair of UV-induced DNA lesions as reported previously (17–19) and suggests that the ability of MC1R signaling to induce NR4A genes is a vital component of this process.

DISCUSSION

The activation of signaling pathways through α-MSH ligand stimulation has long been known to be a key regulator of constitutive and UV-induced pigmentation in humans. The identification of MC1R as the primary target of the α-MSH peptide in melanocytes in the last decade, coupled with genetic association studies, has highlighted the importance of this pathway to mammalian pigmentation (7–9, 50). Despite considerable efforts, the physiological function of MC1R in coordinating melanocyte pigmentation and photo-protection remains poorly understood. The identification of downstream targets and effectors of MC1R represents an important step in advancing our understanding of this pathway. Initial studies reported here reveal the rapid and transient induction of the NR4A family of orphan nuclear receptors following stimulation of B16 mouse melanoma cells with the potent MC1R agonist NDP-MSH. The induction profile of all three members of this receptor family is

negative siRNA, indicating the clearance of UVB-induced CPD lesions by MC1R signaling (Fig. 5C, *upper panels*). Notably, in cells transfected with the combination of NR4A1 and NR4A2 siRNAs there appeared to be little difference in CPD content between vehicle and NDP-MSH-treated melanocytes (Fig. 5C, *lower panels*). Quantification of the fluorescence intensity of these cells from three independent experiments confirmed the attenuated clearance of CPDs by NDP-MSH in cells in which NR4A1/2 expression had been inhibited (Fig. 5D). Specifically, there were significantly lower levels of CPDs detected in negative siRNA-transfected melanocytes following treatment with NDP-MSH compared with vehicle, whereas there was little difference between the levels of CPDs contained in melanocytes in

similar to that observed in other systems, with maximal mRNA expression detected at 1 h for both NR4A1 and NR4A2 receptors, after which expression levels rapidly return to basal levels. Expression of the NR4A3 receptor is similarly induced following NDP-MSH treatment; however, maximal induction is achieved 2 h after MC1R stimulation and returns to basal levels after 4 h. The rapid and transient induction profile observed for these receptors suggests that they are involved in coordinating the immediate transcriptional response of the MC1R signaling pathway. Furthermore, increased NR4A DNA binding activity detected by EMSA coupled with NR4A-responsive reporter assays suggests the NR4A genes are transcriptionally active within the first 24 h post MC1R receptor stimulation.
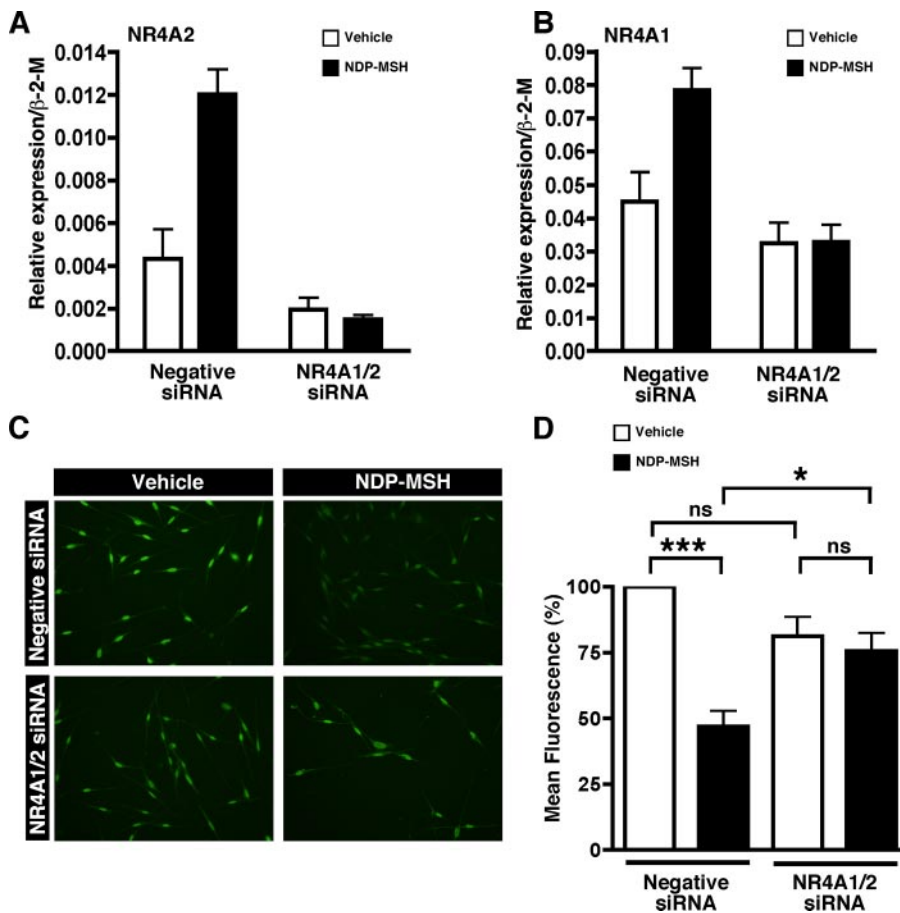
FIGURE 5. **NR4A genes regulate MC1R-induced DNA repair.** *A* and *B*, primary human melanocytes were transfected with either negative or pooled NR4A1 and NR4A2 (*NR4A1/2*) siRNAs. 24 h post transfection, cells were treated with vehicle or 10 nM NDP-MSH and RNA harvested 1 h post treatment. NR4A2 (*A*) and NR4A1 (*B*) expression relative to β-2-microglobulin was determined by real-time PCR. Mean ± S.D. from a representative experiment. *C*, primary human melanocytes were transfected with either negative or pooled NR4A1 and NR4A2 (*NR4A1/2*) siRNAs. 24 h post transfection cells were treated with vehicle or 10 nM NDP-MSH and irradiated with 25 mJ cm^{-2} UVB following a 4-h preincubation in medium containing vehicle or NDP-MSH (10 nM). Nuclear CPD content (*green*) was determined by immunofluorescence. Images are from a representative experiment. *D*, The mean ± S.E. of the relative quantification of three independent immunofluorescence experiments (performed twice using the QF1170 strain and once using the QF1160 strain) using Adobe Photoshop grey scale. Statistical analysis was performed using analysis of variance with a Tukey's post-test (***, $p < 0.001$; *, $p < 0.05$; *ns*, not significant).

We analyzed the effect of specific signaling pathway inhibitors on MC1R-mediated NR4A gene induction in order to identify signaling cascades involved in MC1R signaling via the NR4A receptor family. While inhibition of the cAMP pathway was anticipated to attenuate NR4A induction by MC1R signaling, the ability of the protein kinase C and p38 pathway inhibitors to attenuate NR4A induction by MC1R activation suggests cross-talk between these cascades and MC1R signaling. Similar evidence for multiple signaling pathway involvement in NR4A gene induction has been observed in the context of corticotroph stimulation by corticotrophin-releasing hormone/cAMP (51). Moreover, recent reports have demonstrated cross-talk between MC1R-mediated cAMP and p38/MAPK signaling pathways (41).

Numerous studies have revealed a significant association between variant MC1R and cutaneous malignant melanoma and non-melanoma skin cancer, leading to the conclusion that MC1R is a skin cancer susceptibility gene (3, 18, 52). In light of the links between variant and aberrant MC1R signaling and skin cancer predisposition, it was important to ascertain the

effect of functional variant alleles of MC1R on induction of NR4A. The identification of divergent melanocyte function in the context of WT or variant MC1R proteins will provide vital clues into the mechanisms underlying the association between MC1R status and pigmentation phenotype, UV sensitivity, and melanoma/skin cancer susceptibility. The data presented here reveal that the induction of NR4A gene expression is one of the immediate steps in MC1R signaling in melanocytes expressing wild-type alleles of MC1R. Moreover, in melanocyte strains derived from individuals that are homozygous for the three most common RHC variant MC1R alleles, R151C, R160W, and D294H, the induction of NR4A expression in response to ligand stimulation is compromised. It is possible that the impaired NR4A induction by variant MC1R may underlie to some extent the susceptibility of RHC individuals to skin cancer.

Although much of the cutaneous photo-protective function of the wild-type MC1R protein is undoubtedly attributable to its role in basal and inducible melanization, recent reports suggest a protective role for MC1R against UV-induced DNA damage independent of pigmentation. Human melanocytes carrying common RHC variants were found to have impaired clearance of UV-induced CPD lesions, an effect that was independent of the melanin content of the cells (18). This observation, coupled with previous reports demonstrating the reduction of UV-induced CPD lesions by MC1R signaling (17, 19), suggests that enhancing DNA repair is a vital mechanism by which the MC1R pathway protects melanocytes from UV-induced mutations. Moreover, MC1R-mediated DNA repair in response to UV irradiation is an early event and precedes other MC1R-controlled processes such as melanization.

In light of the fact that NR4A induction by MC1R was an immediate event following receptor stimulation, and functional studies suggested these nuclear receptors were transcriptionally active during the following 24 h, we chose to examine the effect of loss of NR4A expression on DNA repair in the context of MC1R signaling. Melanocytes transfected with a negative control siRNA prior to UVB irradiation exhibited an enhanced rate of DNA repair in response to NDP-MSH stimulation in concordance with previous reports demonstrating MC1R-mediated DNA repair (17–19). Importantly, siRNA knock down of NR4A1 and NR4A2 expression in melanocytes attenuated the

ability of MC1R signaling to repair UVB-induced CPD lesions. These data suggest a novel functional link between MC1R-driven DNA repair and the NR4A nuclear receptor signaling pathway. This contention is further supported by reports suggesting NR4A genes function as sensors of genotoxic stress (35). In these reports NR4A proteins were found to be responsive to the purine anti-metabolite 6-mercaptopurine and its derivatives. The induction of genotoxic stress is a key component of the anti-proliferative activity of this class of compound, leading to the suggestion that the NR4A subgroup mediates the cellular response to genotoxic stress via the activation of an NR4A transcriptional response (35). With the emergence of pharmacological agents capable of targeting the NR4A nuclear receptors (36, 53–55), further understanding of the cytoprotective role of this family may have potential implications in the prevention of melanocytic malignancy.

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